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Identification of the parasite transferrin receptor of *Plasmodium falciparum*-infected erythrocytes and its acylation via 1,2-diacyl-*sn*-glycerol

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ABSTRACT **The transferrin receptor of schizont-infected erythrocytes of *Plasmodium falciparum* (Gambian clone FCR-3/A_2) is a parasite-encoded protein of M_r 102,000, which is present in purified erythrocyte membranes. Polyclonal antiserum to the purified M_r 102,000 protein was raised in rabbits. At physiological pH, immunoaffinity-purified protein bound human ferrotransferrin but not apotransferrin. Conversely, antibody to human transferrin was used to purify the ferrotransferrin–receptor complex from infected cells. The isolated receptor was specifically recognized by the polyclonal rabbit antiserum raised against the M_r 102,000 protein. Preliminary analysis indicated that, unlike the human receptor, the plasmodial transferrin receptor is not a disulfide linked dimer but a single polypeptide acylated via 1,2-diacyl-*sn*-glycerol.**

In vertebrates, transferrin receptors mediate intracellular iron uptake by internalizing and recycling transferrin (1, 2). Binding of iron-loaded transferrin to the receptor results in rapid internalization of the cell surface complex via endocytosis (3). Iron dissociates in an acidic intracellular compartment and the receptor–ligand complex is recycled to the plasma membrane, where it dissociates into free receptor and apotransferrin (4, 5). The receptor is maximally expressed on the surface of actively proliferating cells. Lower levels are detected on resting cells, and terminally differentiated cells, such as mature human erythrocytes, lack the receptor (6–8). The human receptor is an acylated glycoprotein disulfide-linked dimer composed of two M_r 95,000 subunits (9–11).

During its asexual life cycle, the human malaria parasite invades and proliferates in mature host erythrocytes. When intraerythrocytic parasites are deprived of nonheme Fe^{+3} *in vitro*, abnormal growth and cell death are observed (12). Although normal mature erythrocytes are incapable of iron uptake, those infected with *Plasmodium falciparum* internalize ferrotransferrin (13, 32). Gel electrophoresis of parasite proteins bound to ferrotransferrin immobilized on Sepharose showed prominent bands at M_r 93,000 and 200,000 (13). Our studies indicate that a protein of M_r 102,000, synthesized by the intracellular parasite and inserted in the erythrocyte membrane of mature infected cells, is the receptor for serum ferrotransferrin. The purified receptor will not bind transferrin antibodies itself, but can be isolated as a receptor–ferrotransferrin complex using antiserum to human transferrin. Biochemical analysis indicates that the receptor is acylated via 1,2-diacyl-*sn*-glycerol, which may be important for its association with the membrane and regulation in the infected cell (11, 14). A preliminary report of this work has been presented.†

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MATERIALS AND METHODS

Materials. Affi-Gel beads were obtained from Bio-Rad. RPMI 1640 was obtained from GIBCO. Percoll was from Pharmacia. CNBr-activated Sepharose, iodoacetamide, phenylmethylsulfonyl fluoride, *p*-tosyl-L-lysine chloromethyl ketone, pepstatin, leupeptin, and protein A were purchased from Sigma. Phospholipase A_2 (bee venom) and antibodies to human transferrin were obtained from Boehringer Mannheim. Transferrin was from Calbiochem. [^{35}S]Methionine and [^{3}H]myristic acid were from Amersham. Desferal was purchased from CIBA-Geigy.

Isolation of the Erythrocyte Membrane from Schizonts. The *P. falciparum* Gambian clone FCR-3/A_2 was cultured and synchronized as described (15, 16). An 800-μl aliquot of a 50% (vol/vol) suspension of Affi-Gel 731 beads equilibrated in 250 mM sucrose/12.5 mM potassium phosphate, pH 7.2, was added to 400 μl of a washed cell pellet containing 25–30% 36- to 40-hr schizonts. Unbound cells were removed by washing in RPMI 1640 in the presence of 5 mM iodoacetamide/1 mM phenylmethylsulfonyl fluoride/0.1 mM *p*-tosyl-L-lysine chloromethyl ketone/pepstatin at 1 mg/ml/leupeptin at 1 mg/ml. The bound cells were metabolically labeled with [^{35}S]methionine (300 μCi/ml) in a final volume of 1 ml of RPMI 1640 for 45 min at 37°C. They were washed, lysed in 10 vol of lysis buffer (20 mM EDTA/12.5 mM potassium phosphate, pH 7.2, in the presence of protease inhibitors; see above), Vortex mixed for 20 sec, and allowed to settle on ice for a few minutes. The supernatant was discarded, and the bead-bound membranes were washed once in 0.6 M NaCl in lysis buffer. The high salt wash was followed by two washes in 150 mM NaCl/3 M urea/20 mM EDTA/12.5 mM potassium phosphate, pH 7.2. The membranes were extracted from the beads by boiling in an equal volume of double-strength $NaDodSO_4$/PAGE sample buffer. In control experiments, cells were labeled in [^{35}S]methionine at 37°C for 45 min. The cells were washed and lysed by Vortex mixing in an equal volume of lysis buffer. The resulting broken cell fraction was incubated with 200 μl of a 50% (vol/vol) suspension of preequilibrated Affi-Gel beads, and the bound components were washed and extracted as for the sample shown in Fig. 1, lane 3. Erythrocyte membranes from parasitized cells labeled with [^{3}H]myristic acid (16) were also isolated on Affi-Gel beads as described above.

Preparation of Polyclonal Anti-M_r 102,000 Rabbit Antisera. Membranes on beads were prepared as described, from synchronized cultures that were enriched to 85–90% schizonts. The membranes were extracted, and the proteins were electrophoresed on 7.5% acrylamide/$NaDodSO_4$ gels. The Coomassie-stained M_r 102,000 band was cut out (1 mm wide), homogenized and mixed with 2 vol of complete

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Freund's adjuvant. The resulting emulsion was injected into New Zealand White rabbits. Each rabbit was immunized twice with 10 μg of protein and given a final booster shot of 5 μg of protein in incomplete Freund's adjuvant. The antiserum was adsorbed with whole uninfected erythrocytes (5 ml of packed human erythrocytes with 10 ml of rabbit serum) for 30 min at 37°C.

Affinity Purification of the M_r 102,000 Polypeptide and Isolation of the Ferrotransferrin–Receptor Complex. The IgG fraction of the polyclonal rabbit antiserum was prepared (17) and coupled to CNBr-activated Sepharose (18). Ferrotransferrin was prepared as described (19). Schizont-infected cells metabolically labeled with [^{35}S]methionine and [^{3}H]myristic acid (16) were extracted in 0.1% trifluoroacetic acid (20), and the extracts were diluted 1:10 in phosphate-buffered saline (145 mM sodium chloride/5.6 mM sodium monohydrogen phosphate/0.7 mM potassium dihydrogen phosphate, pH 8.0) containing 0.01% bovine serum albumin and 0.02% Nonidet P-40 (dilution buffer). The lysates were applied to the antibody column, and the columns were washed with dilution buffer. The bound proteins were eluted in 50 mM glycine hydrochloride/0.15 M NaCl/0.02% Nonidet P-40, pH 2.8, and precipitated in a final concentration of 5% (wt/vol) trichloroacetic acid at 4°C and analyzed by NaDodSO$_4$/PAGE. When ferrotransferrin–receptor complexes were isolated using immobilized transferrin antibodies, the cell lysates were incubated in the presence of ferrotransferrin. Control incubations were performed in the presence of apotransferrin and of desferal at 5 mg/ml. Chymostatin (50 μg/ml), leupeptin (50 μg/ml), 5 mM iodoacetamide, and pepstatin (10 μg/ml) were present at all stages of the purification procedure.

Analysis of Bound Lipid. [^{3}H]Myristic acid-labeled receptor was either cut out of a gel, electroeluted, and ethanol precipitated (16) or affinity purified as described above. The covalently bound lipid moiety was analyzed as described (16). The amount of noncovalently bound lipid was determined by extraction with chloroform/methanol/water (8:4:3, vol/vol, in the presence of 0.015 M HCl) and was found to be less than 1% of the total counts (Table 1). Base hydrolysis was performed in 0.05 M NaOH/90% (vol/vol) ethanol. Control treatments were carried out in 0.05 M sodium acetate, and less than 1% of the total counts were detected in the organic phase. Bee venom phospholipase A_2 incubations were carried out in 0.1 M Tris·HCl (pH 7.4) containing 0.1% deoxycholate (sodium salt), 1 mM $CaCl_2$, and 20–40 μg of enzyme at 37°C for 1 hr. All treatments were performed with 2000 dpm of substrate. The numbers presented in Table 1 are corrected for background and are an average of three determinations.

NaDodSO$_4$/PAGE and Fluorography. Protein samples were boiled for 5 min in sample buffer containing 2.2% NaDodSO$_4$, 13.3% glycerol, 0.09 M Tris·HCl, 0.1 M dithiothreitol, 0.02% bromophenol blue, pH 6.8. Dithiothreitol was omitted in the nonreduced samples. The samples were loaded on a 7.5% or 10% acrylamide gel with a 5% stacking gel and electrophoresed in 0.025 M Tris·HCl/0.192 M glycine/0.1% NaDodSO$_4$, pH 8.3 (NaDodSO$_4$ running buffer; ref. 16). The radiolabeled protein bands were examined by fluorography using Kodak XAR-5 film.

Table 1. [^{3}H]Fatty acid is covalently bound to the M_r 102,000 protein

Step	[^{3}H]Fatty acid released, %
Chloroform/methanol extraction	
1	0.92
2	0.05
3	<0.01
Base hydrolysis	99.8
Phospholipase A_2	49.2

RESULTS

Isolation of *P. falciparum* Schizont-Infected Erythrocyte Membranes and Analysis of the Associated Parasite Proteins. The method for isolation of cell surface membranes on Affi-Gel 731 beads is essentially that of Jacobson and Branton (21). Parasite proteins from bead-bound intact cells, lysed cells, and washed membranes were analyzed. Comparing lanes 2 and 3 in Fig. 1, a major protein band of M_r 102,000 appears to be quantitatively retained with the bound membranes. Spectrin is not removed under these conditions (data not shown). Hence the M_r 102,000 parasite protein must be stably associated with the schizont-infected erythrocyte membrane, its cytoskeleton, or both. When broken cells were bound to the beads, washed, and extracted, a markedly different protein profile was obtained (Fig. 1, lane 4). The results confirm that the M_r 102,000 component is not merely a major parasite contaminant but a parasite protein specifically associated with the schizont erythrocyte membrane. Uninfected erythrocytes incorporated insignificant amounts of radiolabel and, when analyzed, did not contribute any detectable bands upon NaDodSO$_4$/PAGE and fluorography (data not shown).

Affinity Purification of the M_r 102,000 Protein and Its Binding to Ferrotransferrin. As shown in Fig. 2*A*, lane 4, a major radiolabeled polypeptide of M_r 102,000 was specifically bound by the rabbit antibody. A smaller radiolabeled fragment of M_r ≈45,000 was sometimes detected and was probably due to limited proteolysis of the M_r 102,000 polypeptide. Control columns containing equivalent amounts of IgG from nonimmune rabbits did not bind the M_r 102,000 protein (Fig. 2*A*, lane 3). At physiological pH, the antibody-purified protein formed a high-affinity complex with human ferrotransferrin, which could be quantitatively bound to and eluted from anti-transferrin columns (Fig. 2*A*, lane 5). In the presence of apotransferrin, the M_r 102,000 protein did not

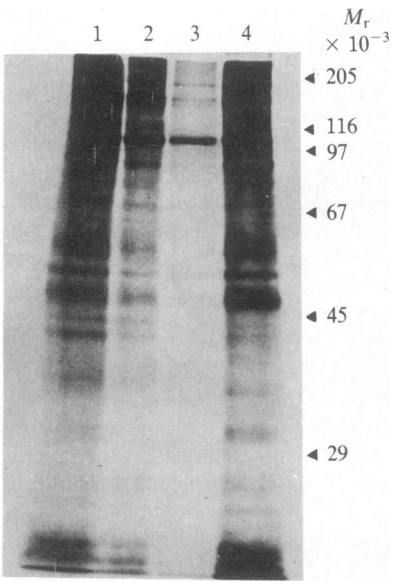

FIG. 1. Comparison of [^{35}S]methionine-labeled parasite proteins from whole cells and erythrocyte membranes bound to Affi-Gel 731 beads. NaDodSO$_4$ extracts were prepared at each step in the isolation of the erythrocyte membrane. Lane 1, whole cells; lane 2, lysed membranes; lane 3, washed membranes; lane 4, broken cells. Molecular weight markers used were as follows: 205, myosin; 116, β-galactosidase; 97, phosphorylase A; 67, albumin (bovine); 45, albumin (egg); 29, carbonic anhydrase.

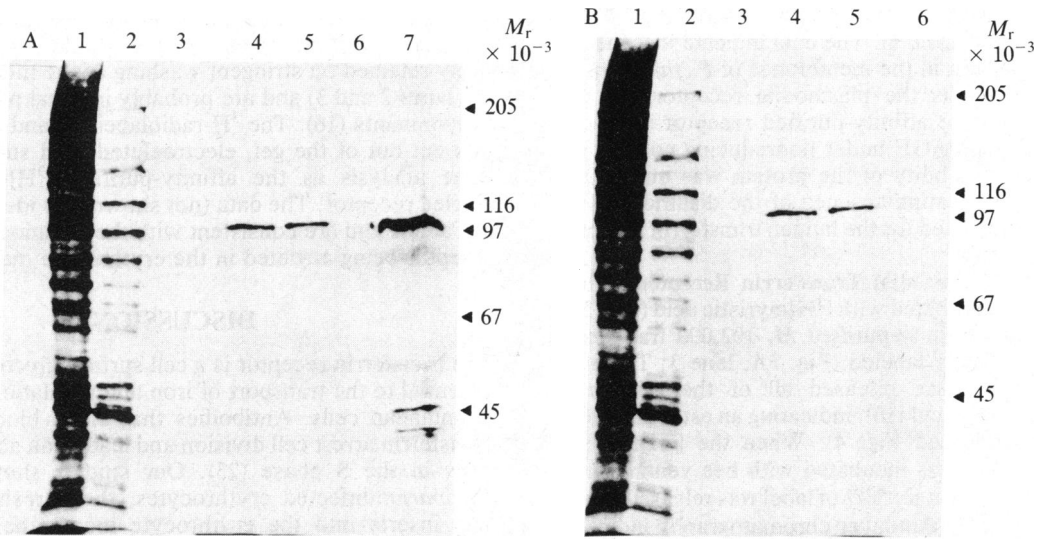

FIG. 2. Affinity purification of [^{35}S]methionine-labeled M_r 102,000 transferrin receptor. (*A*) The 36- to 40-hr schizonts were metabolically labeled with [^{35}S]methionine, and the M_r 102,000 protein was purified using a polyclonal rabbit antibody column. Labeled proteins were electrophoresed on 7.5% polyacrylamide gels and analyzed under reducing conditions by NaDodSO$_4$/PAGE and fluorography. Lane 1, whole cells; lane 2, trifluoroacetic acid extracts; lane 3, protein bound to nonimmune rabbit serum; lane 4, protein bound to immune serum; lane 5, protein from lane 4 bound to an anti-transferrin affinity column in the presence of ferrotransferrin; lane 6, protein from lane 4 bound to an anti-transferrin column in the presence of apotransferrin and desferal; lane 7, protein bound to immune rabbit serum and electrophoresed under nonreducing conditions. Over 90% of the radioactive protein (from lane 4) bound to the anti-transferrin column in the presence of ferrotransferrin. In lanes 5 and 6, equal amounts of the radiolabeled protein were loaded on the anti-transferrin columns. (*B*) [^{35}S]Methionine labeled parasite lysates were applied to anti-transferrin columns to purify the receptor–ligand complex. Lane 1, whole cells; lane 2, trifluoroacetic acid extracts; lane 3, protein bound to anti-transferrin antibodies in the presence of apotransferrin and desferal; lane 4, protein bound to anti-transferrin antibodies in the presence of ferrotransferrin; lane 5, protein from lane 4 bound to polyclonal rabbit antibody; lane 6, protein from lane 4 bound to nonimmune rabbit serum. Over 90% of the counts (protein from lane 4) applied to the rabbit anti-M_r 102,000 protein antibody column were specifically bound and eluted from the column (lane 5). Equal numbers of counts were applied to the columns in lanes 5 and 6. The molecular weight markers are as in Fig. 1.

bind antibody to transferrin (Fig. 2*A*, lane 6), indicating that, at neutral pH, the formation of the ligand–receptor complex required the presence of the ferric ion. Conversely, as shown in Fig. 2*B*, lane 4, antibody to human transferrin was used to purify the preformed high-affinity ferrotransferrin–receptor complex from infected cells. This complex was not formed in the presence of apotransferrin (lane 3). The isolated receptor was specifically recognized by polyclonal rabbit antiserum

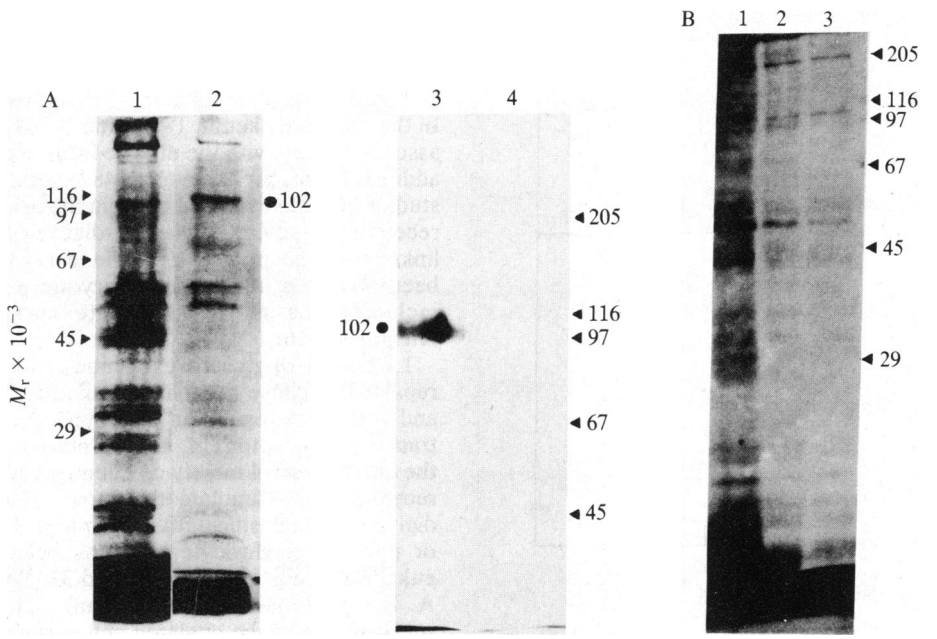

FIG. 3. Acylation of the plasmodial transferrin receptor. (*A*) Fluorogram of the affinity purification of the [^{3}H]myristic acid-labeled M_r 102,000 receptor. Lane 1, whole cells; lane 2, trifluoroacetic acid extracts; lane 3, protein bound to rabbit polyclonal antibody; lane 4, protein bound to nonimmune rabbit serum. Proteins were electrophoresed on a 10% acrylamide gel in lanes 1 and 2 and on a 7.5% acrylamide gel in lanes 3 and 4. Molecular weight markers are as in Fig. 1. (*B*) Fluorogram containing [^{3}H]myristic acid-labeled parasite proteins from whole cells and erythrocyte membranes bound to Affi-Gel beads. Lane 1, whole cells; lane 2, lysed membranes; lane 3, washed membranes. Molecular weight markers are as in Fig. 1.

against the M_r 102,000 protein, but not by nonimmune rabbit serum (Fig. 2*B*, lanes 5 and 6). The data indicate that the M_r 102,000 protein present in the membranes of *P. falciparum*-infected erythrocytes is the plasmodial receptor for ferrotransferrin. When the affinity-purified receptor was analyzed by $NaDodSO_4$/PAGE under nonreducing conditions, the electrophoretic mobility of the protein was unchanged (Fig. 2*A*, lane 7), indicating absence of the disulfide-linked dimeric structure reported for the human transferrin receptor (9, 10).

Acylation of the Plasmodial Transferrin Receptor. When infected cells were incubated with [^{3}H]myristic acid (Fig. 3*A*, lanes 1 and 2), the affinity-purified M_r 102,000 transferrin receptor was covalently labeled (Fig. 3*A*, lane 3; Table 1). Hydrolysis in mild base released all of the associated radiolabel as free fatty acid (16), indicating an ester linkage to the protein (Table 1 and Fig. 4). When the [^{3}H]myristic acid-labeled receptor was incubated with bee venom phospholipase A_2, approximately 50% of label was released (Table 1). Product analysis by thin layer chromatography indicated that the released radioactivity comigrated with free fatty acid (Fig. 4; ref. 16). The evidence suggests that the receptor is acylated via a 1,2-diacyl-*sn*-glycerol moiety, probably linked via a phosphodiester to the protein. Phosphotidylinositol-specific phospholipase C from *Staphylococcus aureus* failed to release any radiolabel from the [^{3}H]myristic acid-labeled receptor (data not shown; ref. 22). In control incubations the phospholipase released 100% of the diglyceride label from the [^{3}H]myristilated-membrane form of the variant surface glycoprotein from *Trypanosoma brucei*. The data suggest the presence of a head group other than unsubstituted inositol, for the putative phospholipid.

Erythrocyte membranes isolated on Affi-Gel beads from parasitized cells labeled with [^{3}H]myristic acid contain a radiolabeled M_r 102,000 band (Fig. 3*B*, lane 3). Two bands of

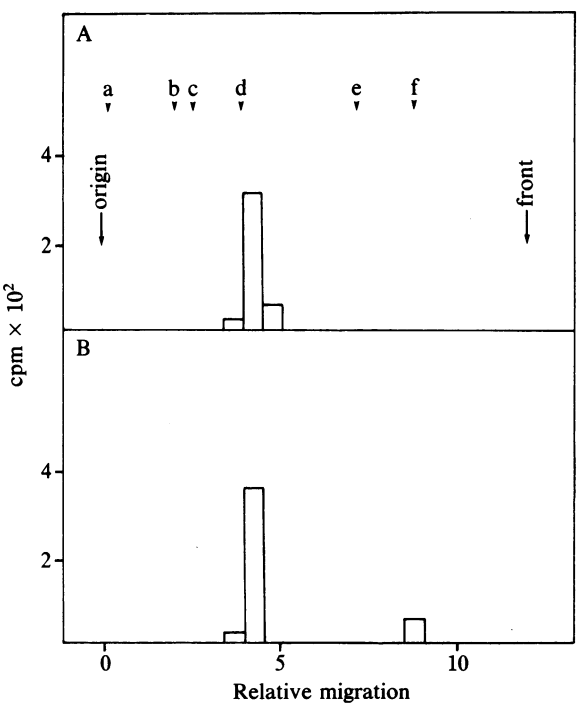

FIG. 4. Product analysis of phospholipase A_2 (*A*) and of base hydrolysis (*B*) treatment of [^{3}H]myristic acid-labeled transferrin receptor. Products were analyzed by thin layer chromatography in petroleum ether/diethyl ether/acetic acid, 80:20:1 (vol/vol) (16). Standards employed were as follows: a, monomyristin; b, 1,2-dimyristin; c, 1,3-dimyristin; d, myristic acid; e, trimyristin; f, ethyl myristate.

M_r 195,000 and 50,000 are also present. However, unlike the M_r 102,000 protein, these additional bands are not quantitatively retained on stringent washing of the membranes (Fig. 3*B*, lanes 2 and 3) and are probably internal parasite protein contaminants (16). The ^{3}H-radiolabeled band of M_r 102,000 was cut out of the gel, electroeluted, and subjected to the same analysis as the affinity-purified [^{3}H]myristic acid-labeled receptor. The data (not shown) are identical to those in Table 1 and are consistent with the plasmodial transferrin receptor being acylated in the erythrocyte membrane.

DISCUSSION

The transferrin receptor is a cell surface glycoprotein that is essential to the transport of iron and regulation of growth in mammalian cells. Antibodies that block binding of serum transferrin arrest cell division and lead to an accumulation of cells in the S phase (23). Our studies show that, in *P. falciparum*-infected erythrocytes, the parasite synthesizes and inserts into the erythrocyte membrane a plasmodial transferrin receptor capable of binding human ferrotransferrin, thus allowing iron uptake by the infected cell. We have detected the receptor on the surface of the infected cell at early schizogony and our data suggest that, in the asexual life cycle of the parasite, this is the peak time of receptor synthesis (K.H., unpublished observation).

The plasmodial receptor has approximately the same mobility as its human counterpart when analyzed by reducing $NaDodSO_4$/PAGE. However, under nonreducing conditions the human receptor appears to be a dimer of M_r 200,000, while the mobility of the parasite receptor is unchanged. Preliminary binding studies using whole cells indicate 60–100,000 parasite transferrin receptors per cell with a single high-affinity binding site for human ferrotransferrin with an association constant of $5 \times 10^7\ M^{-1}$ (data not shown). This is comparable to the cell surface abundance and association constants of transferrin receptors reported in other cell types (7, 19, 24, 25). The human and rat transferrin receptor genes have been cloned (26, 27), and the former has been sequenced (28). A detailed comparison of the structure of the human receptor with that encoded by plasmodium awaits cloning and sequencing of the latter.

Mammalian transferrin receptors contain ester-linked fatty acid, which may be required for their membrane association. In the human leukemic T-cell line CCRF-CEM, fatty acid is associated only with the mature form of the receptor, and its addition can occur as much as 48 hr after synthesis (11). Our studies indicate that in plasmodia the cell surface form of the receptor is acylated via 1,2-diacyl-*sn*-glycerol probably linked by a phosphodiester to the rest of the protein. This has been observed in several eukaryotic proteins (22, 29, 30), including the major merozoite surface protein of *P. falciparum* (16).

1,2-Diacyl-*sn*-glycerol exogenously added to HL-60 cells is reported to cause reversible activation of protein kinase C and the down regulation and hyperphosphorylation of the transferrin receptor (14, 19). In plasmodial cells, release of the diacylglycerol moiety covalently bound to the transferrin receptor may stimulate the same effect. An endogenous diglyceride-releasing activity remains undetected in normal or infected erythrocytes but has been identified in other eukaryotic cells (refs. 29, 31, and 33; Fox, J. A. and Saltiel, A. R., personal communication). Thus binding of ferrotransferrin to the acylated cell surface receptor may activate transient release of the receptor-linked diglyceride signalling internalization of the receptor. This may be key to the regulation and cycling of the receptor in the cell.

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